INVESTIGATION OF THE IN VITRO METABOLISM PROFILE OF A PHOSPHODIESTERASE-IV INHIBITOR, CDP-840: LEADING TO STRUCTURAL OPTIMIZATION

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ABSTRACT:

CDP-840 is a selective and potent phosphodiesterase type IV inhibitor, whose in vitro metabolism profile was first investigated using liver microsomes from different species. At least 10 phase I oxidative metabolites (M1–M10) were detected in the microsomal incubations and characterized by capillary high-performance liquid chromatography continuous-flow liquid secondary ion mass spectrometry (CF-LSIMS). Significant differences in the microsomal metabolism of CDP-840 were found between rat and other species. The major route of metabolism in rat involved para-hydroxylation on the R4 phenyl. This pathway was not observed in human and several other species. The in vitro metabolism profile of CDP-840 was further examined using freshly isolated hepatocytes from rat, rabbit, and human. The hepatocyte incubations indicated more extensive metabolism relative to that in microsomes. In addition to the phase I oxidative metabolites observed in microsomal incubations, several phase II conjugates were identified and characterized by CF-LSIMS. Interspecies differences in phase II metabolism were also found in these hepatocyte incubations. The major metabolite in human hepatocytes was identified as the pyridinium glucuronide, which was not detected in rat hepatocytes. Simple structural modification on R4, such as p-Cl substitution, greatly reduced the species differences in microsomal metabolism. Furthermore, modifications on R3, such as the N-oxide, eliminated the N-glucuronide formation in human. These results not only helped in determining the suitability of animal species used in the preclinical safety studies but also provided valuable directions for the synthetic efforts in finding backup compounds that are more metabolically stable.

Cyclic phosphodiesterase (PDE) enzymes catalyze the hydrolysis of the 3′-phosphoester bonds of cAMP and cGMP to form the corresponding AMP and GMP, and therefore, are involved in controlling the intracellular concentrations of cAMP and cGMP (Torphy, 1998). At least 11 mammalian PDE isoforms have been reported, each encoded by a unique gene. They are distinguished on the basis of their enzyme kinetics, substrate selectivity, and tissue distribution. The type IV family of phosphodiesterases (PDE-IV) is a high-affinity cAMP-selective isozyme, and has been found in almost all cell types that have been implicated in asthma pathogenesis (O’Brien, 1997). Selective PDE-IV inhibitors, therefore, could become promising therapeutic agents for the treatment of asthma and a wide range of other inflammatory diseases (Torphy et al., 1994). CDP-840 ([R–][+1]4–[2(3-cyclopropoxy–4-methoxy phenyl)–2-phenyl ethyl] pyridine) is a potent and selective PDE-IV inhibitor (Hughes et al., 1997; Perry et al., 1998), and was in development for the treatment of asthma. The structure of CDP-840 is shown in Fig. 1.

Drug metabolism studies during the early drug discovery stage are becoming increasingly important. They provide information useful for many aspects of drug discovery, such as drug design, pharmacokinetic evaluation, and toxicity assessment. Biotransformations can be performed in vitro with the microsomal or cytosolic fraction of liver tissue as the enzyme source under the appropriate incubation conditions. Microsomal incubations are useful to pinpoint specific pathways (oxidation or glucuronidation), and can be used to gain an understanding of potential interspecies differences in metabolism (Kumar et al., 1999). Hepatocyte incubations retain phase I and phase II enzyme activities, and therefore, are useful to determine overall metabolism, and mimic in vivo metabolism more accurately than incubations with subcellular fractions (Placidi et al., 1997). In vitro incubations with liver microsomes and/or hepatocytes can be used as a means to predict potential biotransformations in humans and in those species used for preclinical safety studies.

Identification of metabolic pathways of drug candidates significantly relies on innovations in analytical chemistry. The role of mass spectrometry, especially liquid chromatography/mass spectrometry (LC/MS), in drug metabolite identification has become evident and
rapidly characterized by CF-LSIMS, supplemented with the use of hepatocytes prepared from different species. The metabolites were investigated using liver microsomes and Griffith et al., 1993; Li et al., 1995). In this article, the metabolic profiles of CDP-840 were investigated using liver microsomes and HPLC/continuous-flow liquid secondary ion mass spectrometry (CF-LSIMS) is a powerful LC/MS technique for metabolite identification (Nicoll-Griffith et al., 1993). Incubations (0.5 ml) were conducted for 30 min at 37°C and were quenched by the addition of an equal volume of acetonitrile. Precipitated proteins were removed by centrifugation (10,000 rpm; Eppendorf centrifuge model 5415C) for 10 min. Supernatants were analyzed by HPLC/UV and HPLC/CF-LSIMS. Blank incubations containing no drug and control incubations containing boiled microsomes were also conducted at the same time.

Microsomal Oxidative Incubations. Hepatic microsomes were prepared from frozen livers (human, rat, rabbit, ferret, rhesus monkey, etc.) according to a standard procedure (Lu and Levin, 1972). Incubations with microsomes prepared from different species were typically conducted under linear conditions with 80 μM CDP-840 (or CT2412, CT2481) and 0.5 mg of microsomal protein in the presence of a NADPH-generating system as previously described (Nicoll-Griffith et al., 1993). Incubations (0.5 ml) were conducted for 30 min at 37°C and were quenched by the addition of an equal volume of acetonitrile. Precipitated proteins were removed by centrifugation (10,000 rpm; Eppendorf centrifuge model 5415C) for 10 min. Supernatants were analyzed by HPLC/UV and HPLC/CF-LSIMS. Blank incubations containing no drug and control incubations containing boiled microsomes were also conducted at the same time.

Microsomal Glucuronidation. Glucuronidation was achieved using an incubation of 200 μM CDP-840 (or CT2412, CT2481) with 1.0 mg of microsomal protein for 30 min. The incubation was conducted with 12.5 mM MgCl₂, 12.5 mM uridine 5'-diphosphate glucuronic acid (UDPGA) and 20 mM d-saccharic acid-1,4-lactone in a phosphate buffer at pH 6.6. The incubation mixture (0.5 ml) was quenched with an equal volume of acetonitrile. Precipitated proteins were removed by centrifugation for 10 min, and supernatants were analyzed by HPLC/UV and HPLC/CF-LSIMS.

Hepatocyte Incubations. Rat hepatocytes were isolated from male Sprague-Dawley rats by collagenase perfusion of liver as described previously (Silva et al., 1998). Fresh human liver tissue was obtained from consenting donors undergoing partial hepatectomies and from unused liver portions from patients undergoing liver transplants (St. Luc Hospital, Montreal, Canada). The tissues used were morphologically healthy. Human hepatocyte isolation was conducted by a two-step collagenase perfusion of the liver sample as described by Silva et al. (1998). Rabbit hepatocytes were prepared in a similar way to the human hepatocytes. Incubations were conducted with 50 μM CDP-840 (CT2412, CT2481) and 2 × 10⁶ isolated hepatocyte cells/ml. The cell mixture was incubated in Krebs-Henseleit buffer containing 12.5 mM HEPES (pH = 7.4) at 37°C under 95% air and 5% CO₂ for 3 h, and was quenched by the addition of an equal volume of acetonitrile. Precipitated proteins were removed by centrifugation for 10 min. Supernatants were analyzed by HPLC/UV and HPLC/CF-LSIMS.

In Vivo Pharmacokinetics and Metabolism. CDP-840 and CT2412 were administered p.o. at 20 mg/kg in 1% methocel (pH = 2.0) to rats, and were also administered i.v. at 5 mg/kg in saline to rats. CDP-840 was dosed p.o. at 10 mg/kg to rabbits, and 15 mg b.i.d. to humans (Harbinson et al., 1997). The plasma samples obtained postdosing were quenched with an equal volume of acetonitrile, and analyzed by HPLC/UV. Selected samples were analyzed by LC/MS.

Isolation of Metabolites. To prepare ~0.1 mg of metabolites M12 and M17 for NMR characterization, human microsomal incubations in the presence of UDPGA were scaled up appropriately. The isolation was similar to that described previously (Li et al., 1995), using a preparative Waters Novapak C₁₈.

**Fig. 1.** Chemical structure of CDP-840, R₁ + 1,4-12-(3-cyclopentyl-xy-4-methoxy phenyl)-2-phenyl ethyl pyridine, CT2412, and CT2481.

The substructures are labeled as R1 (cyclopentyl), R2 (catechol phenyl ring), R3 (pyridyl), and R4 (phenyl).
column (7.5 × 300 mm), and a Waters 990 diode array detector. Separation of metabolites and parent compound was carried out using a linear gradient of 60% A to 90% A (A = CH3OH; B = 20 mM NH4OAc, pH 5.0) in 30 min at a flow of 4 ml/min. Metabolite peaks were collected manually, concentrated, and desalted using 1 ml of BondElut C18 SPE cartridges, and dried in a Heto-Vac CT110 vacuum centrifuge (Heto Lab Equipment, Berkeroed, Denmark).

**CF-LSIMS.** A JEOL, HX110A double focusing mass spectrometer (EB configuration; JEOL, Boston, MA), equipped with a 10-kV LSIMS source and a cesium ion gun was used in this study. The mass spectrometer was operated in the CF-LSIMS mode, and was described in detail previously (Li et al., 1995). Data acquisition was in positive ion mode and the mass spectrometer was scanned at a rate of 4 s from m/z 0 to 1000 Da. Separation of CDP-840 and its metabolites was achieved using a KAPPA Hypersil BDS C18 (0.30 × 100-mm) capillary column (Keystone Scientific, Bellefonte, PA). The flow rate was 3 μl/min, which was obtained by splitting the main flow (1 ml/min) using a Valco tee (Valco Instruments, Houston, TX). A linear gradient was used from 50% A to 90% A over 40 min (A = methanol, B = 20 mM ammonium acetate adjusted to pH 5.0 with acetic acid; each solvent contained 1.5% glycerol). The incubation supernatant was diluted 5-fold with aqueous ammonium acetate 20 mM adjusted to pH 5.0. The total ion trace displayed an intense molecular ion [M + H]+ at m/z 374 and a minor glycerol adduct [M + H]+ at m/z 466. Three unique fragment ions, A, B, and C, were observed in the fragmentation pattern of CDP-840. Metabolites were similar to that of CDP-840, except for one metabolite, M5. LC/UV profiles of these microsomal incubations presented not quantitative but relative percentages of most of the metabolites generated. The incubation samples were also analyzed using capillary HPLC CF-LSIMS, which did not have UV on-line. The total ion trace has high background consisting of glycerol adduct ions (Li et al., 1995), because glycerol was added to the mobile phase and used as matrix for CF-LSIMS. Reconstructed ion chromatograms of the molecular ions for the parent and all metabolites were therefore presented, as shown in Fig. 2. The reconstructed positive ion chromatograms showed very similar percentage of each metabolite compared with the analytical HPLC/UV traces. The microsomal metabolism profile of CDP-840 in rat differed significantly from other species. At least 10 different metabolites were detected, named M1 through M10. The LSI mass spectra of CDP-840 and three representative phase I metabolites, M5, M6, and M9, are shown in Fig. 3, a–d, and the fragmentation patterns of phase I metabolites are summarized in Table 2.

**Results**

**Oxidative Microsomal Metabolism of CDP-840.** The in vitro metabolism profiles of CDP-840 in humans and potential safety animal species were investigated using microsomal preparations. The incubation mixtures of CDP-840 with microsomal proteins from various species under oxidative conditions were first analyzed by analytical HPLC with UV-photodiode array detection. The percentage of metabolism was calculated using the LC/UV peak area ratio of CDP-840 in an incubation with a control incubation (without NADPH). The rate of microsomal metabolism (nmol/min · mg) of CDP-840 in different species is summarized in Table 1. The UV spectra of the metabolites were similar to that of CDP-840, except for one metabolite, M5. LC/UV profiles of these microsomal incubations presented not quantitative but relative percentages of most of the metabolites generated. The incubation samples were also analyzed using capillary HPLC CF-LSIMS, which did not have UV on-line. The total ion trace has high background consisting of glycerol adduct ions (Li et al., 1995), because glycerol was added to the mobile phase and used as matrix for CF-LSIMS. Reconstructed ion chromatograms of the molecular ions for the parent and all metabolites were therefore presented, as shown in Fig. 2. The reconstructed positive ion chromatograms showed very similar percentage of each metabolite compared with the analytical HPLC/UV traces. The microsomal metabolism profile of CDP-840 in rat differed significantly from other species. At least 10 different metabolites were detected, named M1 through M10. The LSI mass spectra of CDP-840 and three representative phase I metabolites, M5, M6, and M9, are shown in Fig. 3, a–d, and the fragmentation patterns of phase I metabolites are summarized in Table 2.

The positive ion primary mass spectrum of CDP-840 (Fig. 3a) displayed an intense molecular ion [M + H]+ at m/z 374 and a minor glycerol adduct [M + H]+ at m/z 466. Three unique fragment ions, A,
B, and C, at \( m/z \) 281, 213, and 182 were detected. These three fragment ions represented the sequential elimination of methyl pyridyl, cyclopentyl, and methoxy moieties from the protonated molecule (Table 2). Collision-induced dissociation of the molecular ion (B/E linked scan) also produced the same fragment ions (data not shown), and therefore, did not offer any additional structural information. These characteristic fragmentations were subsequently used to determine which of the substructures had undergone metabolism.

The mass spectrum of M1 showed an abundant molecular ion at \( m/z \) 360, which is 14 amu less than that of CDP-840. Two diagnostic fragment ions (A and B) at \( m/z \) 267 and 199 were also shifted 14 amu lower relative to the fragment ions of CDP-840. This implied that M1 was an \( \text{O}^- \)-desmethyl metabolite.

The primary LSI mass spectra of M2 to M9 all showed abundant molecular ions at \( m/z \) 390, i.e., 16 amu higher than that of CDP-840, indicating that they are different mono-oxygenated metabolites. The mass spectrum of M2 showed fragment ions A and B at \( m/z \) 297 and 229, also 16 amu higher than the corresponding fragment ions 281 and 213 of CDP-840, suggesting that the methyl pyridyl and cyclopentyl moieties were not altered. The exact site of hydroxylation could not be assigned based on its LSI mass spectrum or MS/MS spectrum. M2 was speculated to be the monohydroxy metabolite on the C2 position of the ethyl link (Fig. 9), based on coelution and identical mass spectrum with an available authentic standard (racemic). The chirality of this metabolite was not determined.

The primary LSI mass spectra of M3 and M8 were identical, both showed fragment ions A and B at \( m/z \) 281 and 213, suggesting that oxidation occurred on the methyl pyridyl moiety. M8 was confirmed as a monohydroxy metabolite on the C1 position of the ethyl link (Fig. 9), based on coelution and similar mass spectrum with an authentic standard (chiral with C1: S and C2: R). M3 was likely to be the diastereoisomer of M8. A notable minor fragment ion at \( m/z \) 373 was present in the primary mass spectra of M2, M3, and M8, but was not present in the corresponding product ion MS/MS spectra (B/E linked scan) of \([\text{M}+\text{H}]^+\). This fragment ion is postulated to arise from the loss of OH radical from \([\text{M}+\text{H}]^+\) by the initial high-energy ionization process, and seemed to be diagnostic of these two types of hydroxylation.

The primary mass spectrum of M4 showed strong fragment ions A and B at \( m/z \) 297 and 213, indicating that the hydroxylation was on the R1 cyclopentyl group. The exact position of hydroxylation and the stereochemistry could not be determined based on its mass spectrum. Similar mass spectra were obtained for M7 and M9 (Fig. 3d), suggesting that they were either positional isomers or diastereoisomers of M4.

In the primary mass spectrum of M5 (Fig. 3b), fragment ions A and B at \( m/z \) 281 and 213 suggested that oxidation was on the methyl pyridyl moiety. Another abundant fragment ion at \( m/z \) 374 was also
were carried out with hepatocytes isolated from rat, rabbit, and human. The mass spectrum of M11 showed an abundant molecular ion \([M + H]^+\) at \(m/z\) 373. This metabolite was confirmed as the R4 para-phenol, as it coeluted with and showed a mass spectrum identical to the authentic standard.

The primary LSI mass spectrum of M6 (Fig. 3c), a major metabolite in the rat microsomal incubation, showed abundant fragment ions at \(m/z\) 229 and 197, similar to that of M2, except for the absence of a fragment ion at \(m/z\) 373. This metabolite was confirmed as the R4 para-phenol, as it coeluted with and showed a mass spectrum identical to the authentic standard.

The LSI mass spectrum of M10 showed an abundant molecular ion \([M + H]^+\) at \(m/z\) 306, 68 amu less than that of CDP-840. Fragment ion A at \(m/z\) 213 represented the loss of methyl pyridyl from \([M + H]^+\). The spectrum was consistent with the O-desacyclopentyl metabolite, and was confirmed using an authentic standard.

**Metabolism of CDP-840 in Hepatocytes.** Incubations of CDP-840 were carried out with hepatocytes isolated from rat, rabbit, and human livers. The reconstructed positive ion chromatograms (summed MH\(^+\) of CDP-840 and all metabolites) are shown in Fig. 4. The rate of metabolism (nmol/h \cdot 10^6 cells) in these hepatocyte incubations was summarized in Table 1. Both phase I oxidative metabolites (M1–M10) and phase II conjugates were detected. The formation of the phase II metabolites differed significantly between the three species. These phase II metabolites were characterized by CF-LSIMS, and in some cases were also confirmed by NMR. The mass spectral fragmentation patterns of these phase II metabolites are summarized in Table 2.

M11 was present in the rat and rabbit hepatocyte incubations, but not in the human hepatocyte incubation. The primary LSI mass spectrum of M11 showed an abundant molecular ion \([M + H]^+\) at \(m/z\) 536. Fragment ions at \(m/z\) 360, 267, and 199 were observed, and the spectrum below \(m/z\) 400 was identical to that of M1, the O-desmethylnicotinamide metabolite. The mass difference between \([M + H]^+\) (536) and 360 is 176 (dehydrated glucuronic acid), consistent with a glucuronide conjugate. The exact site of glucuronide attachment (either an O-linked or N-linked glucuronide) could not be determined from the LC/MS or LC/MS/MS spectrum.

M2 was detected in both rabbit and human hepatocytes; in fact it was the major metabolite in human hepatocytes. However, rat hepatocytes did not produce M2. The primary LSI mass spectrum of M2 (Fig. 5a) showed an abundant molecular ion at \(m/z\) 550. The spectrum below \(m/z\) 400 was very similar to that of CDP-840, with fragment ions at \(m/z\) 374, 281, and 213. The mass difference between molecular ion (550) and 374 is 176, consistent with a glucuronide conjugate. High-energy collision-induced dissociation of \([M + H]^+\) at \(m/z\) 550 also produced similar fragment ions (data not shown). The pyridine nitrogen is the only functional group in the CDP-840 structure to which a glucuronide could be directly linked; therefore, it was proposed that M2 was a pyridinium glucuronide based on its mass spectrum.

M14 and M15 were present only in the rabbit hepatocyte incubation. The primary LSI mass spectrum of M6 (Fig. 3c) showed an abundant molecular ion \([M + H]^+\) at \(m/z\) 373. This metabolite was confirmed as the R4 para-phenol, as it coeluted with and showed a mass spectrum identical to the authentic standard.

The LSI mass spectrum of M10 showed an abundant molecular ion \([M + H]^+\) at \(m/z\) 306, 68 amu less than that of CDP-840. Fragment ion A at \(m/z\) 213 represented the loss of methyl pyridyl from \([M + H]^+\). The spectrum was consistent with the O-desacyclopentyl metabolite, and was confirmed using an authentic standard.

**Metabolism of CDP-840 in Hepatocytes.** Incubations of CDP-840 were carried out with hepatocytes isolated from rat, rabbit, and human livers. The reconstructed positive ion chromatograms (summed MH\(^+\) of CDP-840 and all metabolites) are shown in Fig. 4. The rate of metabolism (nmol/h \cdot 10^6 cells) in these hepatocyte incubations was summarized in Table 1. Both phase I oxidative metabolites (M1–M10) and phase II conjugates were detected. The formation of the phase II metabolites differed significantly between the three species. These phase II metabolites were characterized by CF-LSIMS, and in some cases were also confirmed by NMR. The mass spectral fragmentation patterns of these phase II metabolites are summarized in Table 2.

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M14 and M15 were present only in the rabbit hepatocyte incubation. The primary LSI mass spectra exhibited abundant molecular ions \([M + H]^+\) at \(m/z\) 566. The mass spectrum of M15 (Fig. 5b) below mass 400 amu resembled that of M9, the monohydroxy cyclopentyl metabolite. M15 was postulated to be the glucuronide conjugate of a hydroxy cyclopentyl, although the possibility of a hydroxy cyclopentyl, pyridinium glucuronide could not be ruled out. M14 was very minor, and its mass spectrum was too weak to produce useful fragment ions. It was likely an isomer of M15.

Metabolite M19 was also formed only in the rabbit hepatocyte incubation. Ions at \(m/z\) 386 and 403 corresponded to \([M + H]^+\) and \([M + NH_4]^+\). Fragment ions at \(m/z\) 213 and 306 were detected, suggesting that the cyclopentyl group was lost. The mass difference between \([M + H]^+\) 386 and fragment ion 306 was 80, implying a sulfate conjugate (Fig. 10).

Several metabolites such as M6, M13, M16, M17, M18, and M20 were detected in the rat hepatocyte incubation, but not in the human hepatocyte incubation. The mass spectrum of M17 showed abundant molecular ions \([M + H]^+\) at \(m/z\) 566. The spectrum below mass 400 amu was identical to that of M6, the para-phenol metabolite. M17 was, therefore, identified as R4 para-hydroxy glucuronide conjugate (Fig. 5c).

The mass spectrum of M13 (data not shown) displayed a strong \([M + H]^+\) at \(m/z\) 408, 34 amu higher than that of CDP-840. Fragment ions at \(m/z\) 390, 297, and 229 were detected, consistent with a dihydrodiol on the R4 phenyl. The mass spectrum of M16 (data not
shown) showed a molecular ion [M + H]⁺ at m/z 406, 32 amu higher than that of CDP-840, indicating a dioxygenated metabolite, and was proposed to be an R4 catechol.

Metabolite M6', which eluted very closely with M6, showed a protonated molecule at m/z 420, and major fragment ions at m/z 327 and 259. The LSI mass spectrum of M6' exhibited a very similar pattern to that of M6, except that all the ions were shifted 30 amu higher. The increment of 30 amu could imply an additional methoxy group on the R4. This metabolite was proposed to be the R4 O-methyl catechol.

Metabolite M18 showed abundant molecular ion at m/z 596, the LSI mass spectrum exhibited a very similar pattern to that of M17, except that all the ions were shifted 30 amu higher. The increment of 30 amu would again imply an additional methoxy group on the R4 phenyl (see structure in Fig. 10).

The LSI mass spectrum of metabolite M20 displayed a strong [M + H]⁺ at m/z 697. Fragment ions at m/z 229, 281, 297, 374, and 390 were detected. The spectrum suggested that M20 was a glutathione adduct (Fig. 5d).

**Glucuronidation of CDP-840 in Microsomes.** A proposed pyridinium glucuronide metabolite M12 was detected in rabbit and human hepatocytes. If the proposed structure is correct, the same metabolite might be formed in the incubation of CDP-840 with microsomal proteins from various species in the presence of UDPGA. Indeed, M12 was detected in the incubations with human, rabbit, rhesus monkey, and guinea pig microsomes in the presence of UDPGA at rates of 1.23, 0.17, 0.03, and 0.02 nmol/min ⋅ mg of protein. This metabolite was not detected in the incubations with rat, dog, mouse, or ferret microsomes, nor was it not detected in the rat hepatocyte incubation.

M12 was isolated from the human microsomal incubation by semi-preparative HPLC. The structure was identified as β-linked pyridinium glucuronide (Fig. 6; Table 3) by NMR. The linkage between the glucuronide anomeric carbon and the pyridyl nitrogen was confirmed by long-range correlation experiments. In particular, a three-bond correlation between the glucuronide anomeric proton (H-26) and the two carbons adjacent to the pyridyl nitrogen (C-11 and C-12) was observed. In addition, the reverse correlation between the two protons adjacent to the pyridyl nitrogen (H-11 and H-12) and the glucuronide anomeric carbon (C-26) was also observed, confirming the presence of the pyridinium glucuronide species depicted in Fig. 6. A coupling constant of 8.6 Hz was observed at the anomeric proton, indicating that the glucuronide linkage has the β configuration.

When the authentic standard of M6 (R4 para-phenol) was incubated with rat microsomal protein in the presence of UDPGA, a metabolite was also detected. This metabolite had the same retention time and LSI mass spectrum as those of M17 in rat hepatocytes. M17 was isolated from the microsomal incubation by prep-HPLC, and NMR studies confirmed its structure as R4 para-O-glucuronide with the configuration.

**In Vitro Metabolism of CT2412 and CT2481.** CT2412 and CT2481 are two synthetic analogs (see structures in Fig. 1) of CDP-840. Both have a para-Cl substitution on the R4 phenyl, and CT2481 also has a simple modification on R3, i.e., N-oxide. The in vitro metabolism profiles of these two analogs were investigated using microsomal preparations from rat and rhesus monkey. The microsomal oxidative metabolism profiles of CT2412 in rat and rhesus monkey are shown in Fig. 7. The profiles were clearly similar in the two species. The metabolites were identified analogously to those described for CDP-840 metabolites. They were descyclopentyl (1), hydroxy cyclopentyl (2 and 3), N-oxide (4), and desmethyl (5) metabolites. When CT2412 was incubated with microsomes under glucuronidation conditions, the N-glucuronide metabolite of CT2412 was detected in human microsomes (3%) and rabbit microsomes (3%), but to a lesser extent compared with that of CDP-840. The microsomal oxidative metabolism profiles of CT2481 in rat and rhesus monkey are shown in Fig. 7. The profiles were clearly similar in the two species. The metabolites were identified analogously to those described for CDP-840 metabolites. They were descyclopentyl (1), hydroxy cyclopentyl (2 and 3), N-oxide (4), and desmethyl (5) metabolites. When CT2412 was incubated with microsomes under glucuronidation conditions, the N-glucuronide metabolite of CT2412 was detected in human microsomes (3%) and rabbit microsomes (3%), but to a lesser extent compared with that of CDP-840. The microsomal oxidative metabolism profiles of CT2481 in rat and rhesus monkey were also identical (data not shown), with descyclopentyl, hydroxy cyclopentyl, and desmethyl being the major metabolites. The reduction of N-oxide to free pyridyl was also observed, but was very minor based on LC/UV and LC/MS data. In the glucuronidation studies, the
N-glucuronide metabolite was not detected for CT2481 in human or rabbit microsomes supplemented with UDPGA.

The metabolism profiles of CT2412 and CT2481 in rat hepatocytes were also investigated, and were similar to those in microsomes with addition of phase II glucuronide conjugates (1-gluc, data not shown). The phase II metabolism, however, was significantly reduced compared with that of CDP-840 in rat hepatocytes.

In Vivo Pharmacokinetics of CDP-840 and CT2412. Rat plasma concentrations of CDP-840 and CT2412 following p.o. or i.v. dosing were determined by HPLC with UV detection, and were plotted in Fig. 8. The levels of CDP-840 in rat plasma after 20-mg/kg p.o. dosing were not detectable. In the i.v.-dosed (5 mg/kg) rats, CDP-840 had a plasma concentration of 4.4 µM (average of three rats) at 5-min postdosing, and no detectable levels of CDP-840 were found after 2 h. In healthy male volunteers at 16 mg b.i.d dosing with CDP-840, the half-life of CDP-840 was determined to be 6 h. For CT2412, plasma levels in p.o.-dosed rats were detected with a Cmax of 0.44 µM at 2 h. In i.v.-dosed rats, a similar concentration (3.7 µM) of CT2412 at 5-min postdosing was observed compared with that of CDP-840, however, the levels of CT2412 could be detected up to 4 h (0.3 µM) postdosing.

In Vivo Metabolism of CDP-840. The plasma samples obtained after p.o. dosing to rat, rabbit, and human were analyzed. The major circulating metabolites observed in rat plasma were R4 para-phenol (M6) and N-oxide (M5). In rabbit plasma, the hydroxy cyclopentyl (M9) was the major metabolite. In human plasma, pyridinium glucuronide (M12), N-oxide (M5) and hydroxy cyclopentyl (M9) were detected as major metabolites.

Discussion

The in vitro metabolism profiles of CDP-840 were evaluated using hepatic microsomes and freshly isolated hepatocytes from different species. With the combination of HPLC/UV, LC/MS, NMR, and available synthetic standards, in vitro phase I and phase II metabolites of CDP-840 were characterized. Primary CF-LSI mass spectra of CDP-840 and its metabolites all exhibited abundant molecular ions and unique fragment ions, allowing the sites of metabolism to be easily and quickly pinpointed to a particular substructure. NMR data and synthetic standards provided confirmation of metabolite structures. In our study of CDP-840 metabolism, many synthetic standards had been or were rapidly synthesized, making some of the metabolite identification much more straightforward.

The phase I oxidative metabolism profile of CDP-840 was evaluated using microsomal proteins from various species. Up to 10 oxidative metabolites were detected. The reconstructed CF-LSIMS ion chromatograms of all MH+ of metabolites and CDP-840 showed very

| Table 3

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<th>Position</th>
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<td>8′</td>
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*Chemical shifts are relative to dimethyl sulfoxide (2.49 ppm).
similar profiles and peak ratios as the analytical HPLC/UV traces. The pyridyl nitrogen is likely the site for protonation, and therefore the CF-LSIMS responses for the metabolites and CDP-840 were believed to be very similar. The sum of the reconstructed ion chromatogram (Fig. 2) reflected not quantitative but relative amount of metabolites generated. Several sites of CDP-840 were found to undergo biotransformation. The R1 cyclopentyl group was the major site for metabolism in most species. Several hydroxy cyclopentyl metabolites were generated. Several sites of CDP-840 were found to undergo biotransformation. The R2 catechol ring, both desmethyl and descyclopentyl metabolites were observed. Similar biotransformations were also reported for rolipram (Krause and Kuhne, 1992, 1993), a PDE-IV inhibitor from Schering, which has the same catechol moiety. On the R3 pyridyl, N-oxidation was detected, and on the R4 phenyl, para-hydroxylation was observed. The oxidative metabolism of CDP-840 in human microsomes was similar to those in hepatic microsomes from rhesus monkey, guinea pig, dog, ferret, rabbit, and mouse, although the relative percentages of each metabolite varied in these different species. The metabolism profile in rat microsomes, however, differed significantly from these species. The major site of metabolism in rat involved the para-hydroxylation (M6) on the R4 phenyl ring. M6 was detected as a minor metabolite in rabbit microsomes, and was not detected in the microsomal incubations of CDP-840 with human and other species. The oxidative microsomal metabolism pathways of CDP-840 are summarized in Fig. 9.

Phase II metabolism of CDP-840 was evaluated using hepatocytes. Freshly isolated hepatocytes retain phase I and phase II enzyme activities, and therefore, should provide a better correlation with in vivo metabolism (Placidi et al., 1997; Nicoll-Griffith et al., 1999). Rat hepatocytes are used as our primary in vitro system for checking overall metabolic stabilities of synthetic compounds at the early drug discovery stage, and proved to be very useful to determine the overall metabolism (both phase I and phase II simultaneously). Because significant interspecies difference in metabolism were found between rat and other species from microsomal studies, hepatocytes isolated from different species were prepared, and their metabolism profiles were compared. Metabolism profiles of CDP-840 in rat, rabbit, and human hepatocytes indicated more extensive metabolism relative to that in microsomes, and provided evidence of additional interspecies differences in the phase II metabolism. The in vitro phase II metabolism pathways of CDP-840 are summarized in Fig. 10.

In the human hepatocyte incubation of CDP-840, the phase II metabolism dominated, and the major metabolite was confirmed as R3 pyridinium glucuronide (M12). It was also found to be the major circulating metabolite of CDP-840 in human plasma. This metabolite was not detected in the rat hepatocyte incubation. The formation of pyridinium glucuronides has been reported in the literature for triphenlenamine (Yeh, 1991) and nicotine (Byrd et al., 1992). The food pyrolysis product 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine also forms pyridinium glucuronides in human and rabbit microsomes under UDPGA, but not in rat microsomes (Styczynski et al., 1993).

The metabolism profile of CDP-840 in rat hepatocytes was very different from that in human hepatocytes. The extent of metabolism was also greater in rat compared with that in human or rabbit. Several metabolites (M6', M13, M16, M17, M18, and M20) were detected in rat (some were also in rabbit) hepatocytes, but were not present in human hepatocytes. These metabolites were probably formed through an epoxide intermediate on the R4 phenyl. The epoxide could isomerize nonenzymatically to the R4 para-phenol (M6). It could also convert to a R4 dihydrodiol (M13) possibly by epoxide hydrases. The stable diol could further dehydrate enzymatically to yield the para-phenol (M6), or be oxidized by a dehydrogenase to generate a diphenolic metabolite (M16). The epoxide could also be susceptible to conjugation (M20) with glutathione by glutathione S-epoxide transferase. The formation of aromatic epoxides and further metabolized products such as phenols, dihydrodiols, and glutathione conjugates is well documented in the literature, as in the case of naphthalene (Jerina et al., 1970) and monohalogenobenzenes (Parke, 1968). The R4 diphenolic metabolite (M16) could undergo O-methylation to form M6'. O-Methylation of catechol metabolites formed by the epoxide-dihydrodiol pathway is in the literature for diphenylhydantoin (Glazko, 1973). The reaction was mediated by catechol O-methyl transferase (Bakke, 1970). Both M6' and M6 (R4 para-phenol) could
also further conjugate with glucuronic acid to form the corresponding glucuronides M17 and M18.

In the development of any potential therapeutic drug, the preclinical safety studies are crucial to evaluate efficacy, pharmacokinetics, and toxicity. It is very important that the safety species produce the same metabolites as those found in humans. Rats are typically used as the early preclinical safety species. The profound interspecies differences in the in vitro metabolism of CDP-840 between rat and human would make the preclinical safety studies in the rat invalid. Species differences in metabolism became a critical issue, and one of the important criteria to select potential backup compounds was to eliminate these differences. CT2412 was quickly identified, it has similar potency against PDE-IV (GST-met248A assay: 6.7 nM) compared with that of CDP-840 (4.3 nM). The para-Cl substitution on R4 eliminated the R4 para-phenol metabolite and other epoxide-mediated metabolites in rat. This simple substitution improved the metabolic stability in rat, and most importantly, similar metabolism profiles of CT2412 were observed in the microsomal incubations with rat and rhesus monkey.

In the in vivo pharmacokinetic studies of CDP-840 and CT2412 in rats, it was found that CDP-840 had extremely poor bioavailability in rats (0%). No plasma levels of CDP-840 were detected in the p.o.-dosed rats, and CDP-840 also had very short half-life in the i.v.-dosed rat. These results are consistent with the fact that CDP-840 was extensively metabolized in rat from the in vitro studies. In healthy male volunteers with 16 mg of CDP-840 dosed b.i.d., the half-life was determined to be 6 h; this is consistent with the in vitro results, which showed less metabolism of CDP-840 in human than in rat. For CT2412, the improvement in metabolic stability in vitro was also reflected in the pharmacokinetics in vivo. CT2412 has improved bioavailability (11%), and longer half-life in rats. The analysis of plasma samples obtained after p.o. dosing of CDP-840 to rat, rabbit, and human showed that the predominant metabolites detected in plasma were also the major metabolites formed in the hepatocyte incubations. A good correlation between the in vitro and in vivo metabolism was established. In vitro techniques, especially the hepatocyte incubations can be therefore used to predict the in vivo metabolism of potential backup compounds in animal models and in humans.

In summary, the extent of metabolism and significant interspecies differences in metabolism were the two main issues for CDP-840. The in vitro metabolism studies described here not only helped in determining the suitability of animal species used in preclinical safety studies but also were extremely useful in identifying backup compounds and directing the synthetic efforts. Simple structural modifications on CDP-840, such as R4 para-Cl substitution and R3 N-oxide, greatly improved the metabolic profile and stability. As a result, metabolism guided studies have been greatly accelerated for lead finding and optimization of drug candidates.

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References


